

FORM PTO-1390 (Modified)
(REV 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

10690//126885

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)

09/937924

INTERNATIONAL APPLICATION NO
PCT/JP00/02053INTERNATIONAL FILING DATE
March 30, 2000PRIORITY DATE CLAIMED
April 2, 1999

TITLE OF INVENTION

SILVER CONJUGATED PROTEIN, AND ANTIBACTERIAL/ANTIFUNGAL AGENT AND ANTIBACTERIAL/
ANTIFUNGAL PAPER USING THE SAME

APPLICANT(S) FOR DO/EO/US

Yoshihiro Kawaguchi

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2))
7. ☒ A copy of the International Search Report (PCT/ISA/210)
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made, however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)) (Unexecuted)
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409)
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter
19. ☒ Certificate of Mailing by Express Mail
20. ☐ Other items or information.

- 1) Transmittal Letter;
- 2) Check in the amount of \$680.00 representing the filing fee and recording fee for Assignment;
- 3) 10 Sheets of drawings; and
- 4) Return postcard

Katayama Chemical, Inc. owner of all right, title and interest, claims small entity status under 37 CFR §1.27.

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|--|--|--|--|--|--|
| U.S. APPLICATION NO. 09/937924 | | INTERNATIONAL APPLICATION NO. PCT/JP00/02053 | | ATTORNEY'S DOCKET NUMBER 10690//126885 | |
|--|--|--|--|--|--|

21. The following fees are submitted:

| | | | | | |
|--|--------------|--------------|-----------|-------------------------------------|-----------------|
| BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00 <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 <p style="text-align: center;">ENTER APPROPRIATE BASIC FEE AMOUNT =</p> | | | | CALCULATIONS PTO USE ONLY | |
| | | | | \$890.00 | |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)) | | | | \$130.00 | |
| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
| Total claims | 11 - 20 = | 0 | x \$18.00 | \$0.00 | |
| Independent claims | 1 - 3 = | 0 | x \$80.00 | \$0.00 | |
| Multiple Dependent Claims (check if applicable). | | | | <input type="checkbox"/> | \$0.00 |
| TOTAL OF ABOVE CALCULATIONS = | | | | \$1,020.00 | |
| Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). | | | | <input checked="" type="checkbox"/> | \$510.00 |
| SUBTOTAL = | | | | \$510.00 | |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)) | | | | \$130.00 | + |
| TOTAL NATIONAL FEE = | | | | \$640.00 | |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). | | | | <input checked="" type="checkbox"/> | \$40.00 |
| TOTAL FEES ENCLOSED = | | | | \$680.00 | |
| | | | | Amount to be: refunded | \$ |
| | | | | charged | \$ |

☒ A check in the amount of **\$680.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **02-4467** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Warren K. MacRae
 NAME

37,876
 REGISTRATION NUMBER

October 2, 2001
 DATE

1144 47

09/9,379,24

JC12 Rec'd PCT/PTO 0-2 OCT 2001

Express Mail Label No. EL715972196US

Docket No.: 10690/126885

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re National Application of :

Yoshihiro KAWAGUCHI

International Appln. No. PCT/JP00/02053

International Filing Dated: March 30, 2000

Filed Concurrently Herewith On October 2, 2001

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)

Examiner: Not Yet Known

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Art Unit: Not Yet Known

For: **SILVER CONJUGATED PROTEIN, AND
ANTIBACTERIAL/ANTIFUNGAL AGENT
AND ANTIBACTERIAL/
ANTIFUNGAL PAPER USING THE SAME**

New York, New York

October 2, 2001

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents

Box PCT

Washington, DC 20231

Sir:

Please amend the above-identified application as follows:

In The Claims

Please amend claim 8 as follows:

--8. An antibacterial/antifungal agent containing a silver conjugated protein as
set forth in claim 1 as an active component. --

$\frac{1}{2} \times \frac{1}{2} = \frac{1}{4}$

Respectfully submitted,


Warren K. MacRae

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Docket No.: 10690/126885

MARKED-UP COPY OF AMENDED CLAIMS

8. An antibacterial/antifungal agent containing a silver conjugated protein as set forth in [any one of claims 1 to 7] claim 1 as an active component.

Applicant or Patentee: Katayama Chemical, Inc. Attorney's 10690/126885
 Serial or Patent No.: _____ Docket No.: _____
 Filed or Issued: _____
 For: Silver Conjugated Protein, and Antibacterial/Antifungal Agent and Antibacterial/Antifungal
Paper using the same

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
 STATUS (37 CFR 1.9 (f) and 1.27 (c)) — SMALL BUSINESS CONCERN

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Katayama Chemical, Inc.

ADDRESS OF CONCERN 2-10-15, Higashiawaji, Higashiyodogawa-ku,
Osaka-shi, Osaka 533-0023 Japan

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9 (d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled Silver Conjugated Protein,
and Antibacterial/Antifungal Agent and Antibacterial/Antifungal Paper using the same by inventor(s)
Yoshihiro KAWAGUCHI described in

☒ the specification filed herewith

☐ application serial no. _____, filed _____

☐ patent no. _____, issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9 (d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____

ADDRESS _____

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Hirohiko KATAYAMA

TITLE OF PERSON OTHER THAN OWNER _____

ADDRESS OF PERSON SIGNING 1591-3-201, Hirano, Mikage, Mikage-cho, Higashinada-ku, Kobe-shi,
Hyogo, JAPAN

SIGNATURE _____

Hirohiko KATAYAMA

DATE September 26, 2001

10/PRTS

09/937924

DESCRIPTION

JC12 Rec'd PCT/PTO 02 OCT 2001

Silver Conjugated Protein, and
Antibacterial/Antifungal Agent and Antibacterial/Antifungal
Paper using the same

5

Technical Field

The present invention relates to a novel silver
conjugated protein having antibacterial/antifungal activity and
an antibacterial/antifungal agent and an antibacterial/
10 antifungal paper using the same.

Background Art

In recent years, from the viewpoint of health and
sanitation, a lot of medical apparatuses and instruments,
15 stationery, textiles, paper products, daily-use goods, bath goods
and the like are antibacterially/antifungally treated for
preventing growth of various species of microorganisms such as
fungi and bacteria. For example, hospitals use plastic
stationery and instruments which are provided with
20 antibacterial/antifungal activity for the purpose of preventing
nosocomial infection. Now higher safety for health is required
of such articles that directly contact human bodies and food,
and there is a demand for development of an antibacterial/
antifungal agent with excellent antibacterial/antifungal activity
25 and products treated with the agent.

As such antibacterial/antifungal agents, there are
 proposed antibacterial/antifungal agents wherein antibacterial
 /antifungal metals such as silver, copper and zinc are adsorbed
 on water-insoluble scleroproteins (which are proteins contained
 5 in eggshell membrane, feathers, wool, silk and the like; and
 collagen, elastin, silk fibroin and the like separated from such
 materials) (see Japanese Unexamined Patent Publication Nos.
 HEI 6(1994)-65013, HEI 8(1996)-188513 and HEI 8(1996)-
 258235).

10 These antibacterial/antifungal agents are obtained, for
 example, by mixing and stirring an eggshell membrane powder
 with an aqueous solution of silver nitrate, subsequently filtering
 the precipitate out of the resulting mixture solution, and then
 washing the precipitate with water, followed by dehydrating and
 15 drying (see Japanese Unexamined Patent Publication No. HEI
 6(1994)-65013, paragraph [0011], for example).

However, the antibacterial/antifungal agents thus
 obtained have not been able to provide a sufficient antibacterial
 /antifungal activity to a variety of articles. It is considered
 20 that the reason is that the antibacterial/antifungal agents have
 low contents of the antibacterial/antifungal metals, and that
 the binding between the proteins and the antibacterial/
 antifungal metals is weak physical or ionic binding and therefore
 the antibacterial/antifungal metals are easily released from the
 25 proteins owing to water-washing and the like.

On the other hand, Japanese Pharmacopoeia describes silver protein, a compound of silver and protein. The compound of silver and protein exists as a peptide aggregate composed of water-insoluble Ag conjugated peptides which have undergone a secondary structural change owing to their binding to silver ions and water-soluble peptides which do not combine easily to silver ions because the water-soluble peptides do not have intramolecular thiol groups.

Accordingly, if the silver protein is used for other applications than medical supplies, the silver protein elutes easily and cannot exhibit a sufficient effect as an antibacterial /antifungal agent.

In addition, a number of inorganic metal compounds containing silver, copper, zinc and the like have been developed as active components for antibacterial /antifungal paper. The mechanism of the activity of these inorganic metal compounds is not well known. However, it is considered that the active components absorbed as metal ions in microbial cells inhibit the fundamental metabolism in the respiration and the electron transfer sequence of microorganisms or the transfer of substances through cell membrane of microorganisms and thereby exhibit the antibacterial /antifungal activity. However, a problem lies in that active materials composed of the inorganic metal compounds can hardly be applied (fixed) onto paper without using a resinous binder.

An object of the present invention is to provide a water-insoluble conjugated protein which contains a high content of silver and from which antibacterial/antifungal silver is not released easily, and an antibacterial/antifungal agent and an antibacterial/antifungal paper using the conjugated protein.

Disclosure of Invention

The present invention provides a water-insoluble silver conjugated protein formed of a silver salt and a water-soluble protein containing 0.1 to 200 μ moles of active thiol groups per gram.

The present invention also provides an antibacterial/antifungal agent containing the above-mentioned silver conjugated protein as an active component.

The present invention also provides an antibacterial/antifungal paper treated with the above-mentioned antibacterial/antifungal agent.

Brief Description of Drawings

Fig. 1 is a graph showing a relationship between the concentration of an aqueous solution of silver nitrate in forming a silver conjugated protein in accordance with the present invention and the yield of the obtained silver conjugated protein as well as the content of silver in the obtained silver conjugated

protein (Preparation Example 10);

Fig. 2 is a graph showing a relationship between the concentration of an aqueous solution of silver nitrate in forming a silver conjugated protein in accordance with the present invention and the yield of the obtained silver conjugated protein as well as the content of silver in the obtained silver conjugated protein (Preparation Example 11) ;

Fig. 3 shows the results of a fungal resistance test on a silver conjugated protein ② in accordance with the present invention (Test Example 9);

Fig. 4 shows the results of a fungal resistance test on a silver conjugated protein ③ in accordance with the present invention (Test Example 9);

Fig. 5 shows the results of a fungal resistance test on a known silver-containing antibacterial agent (Zeomic) (Test Example 9);

Fig. 6 shows growth of a fungus where the silver conjugated protein ③ of the present invention is applied on liner paper at a coating weight of 0.14g/m² (Test Example 10);

Fig. 7 shows growth of a fungus where the silver conjugated protein ③ of the present invention is applied on liner paper at a coating weight of 0.42 g/m² (Test Example 10);

Fig. 8 shows growth of a fungus where the silver conjugated protein ③ of the present invention is applied on liner paper at a coating weight of 0.14g/m² (Test Example 12);

Fig. 10 shows the surface of copy paper coated with the silver conjugated protein ① of the present invention (Test Example 13);

Fig. 11 shows the surface of copy paper coated with the silver conjugated protein ③ of the present invention (Test Example 13); and

Fig. 12 shows the surface of copy paper coated with the known silver-containing antibacterial agent (Zeomic) (Test Example 13).

Best Mode for Carrying Out the Invention

The "active thiol group" of the protein used in the present invention means a mercapto group (-SH) which is capable of reacting easily with an aqueous solution of a silver compound to form a Ag - S bond.

The content of the active thiol groups in the protein of the present invention is 0.1 to 200 μ mol/g, preferably 5 to 100 μ mol/g with respect to the weight of the protein.

A content of the active thiol groups smaller than 0.1 μ mol/g is not preferable because only a small amount of silver binding to the active thiol groups, and therefore, a desirable water-insoluble silver conjugated protein cannot be obtained.

Also, a content of the active thiol groups larger than 200 μ mol/g is not preferable because a water-insoluble conjugated compound having a localized binding of silver to the active thiol groups may precipitates. That is, this phenomenon results in a decrease in the amount of silver binding.

The content of the active thiol groups can be determined as L-cysteine equivalent by a DTNB method (Ellman's method) by preparing an aqueous solution of protein quantified beforehand (see Biochemical Experiment 10 "Quantitative Determination of SH group," published by the Academic Publication Center, 1981, p.p. 86 to 93).

The "water-soluble protein having an active thiol group content of 0.1 to 200 μ mol/g" used in the present invention is not particularly limited so long as the content of the active thiol groups is within the above-mentioned range. Examples thereof include whey protein, a hydrolysate of whey protein, a water-solubilized product of whey protein, a hydrolysate of eggshell membrane protein and a water-solubilized preparation of eggshell membrane protein. These can be suitably used.

The above-mentioned water-soluble proteins retain an emulsification property which is a characteristic of starting proteins. Antibacterial/antifungal agents prepared from these water-soluble proteins are expected to have good adherence to an object.

It should be understood that the "water-solubility" of

the water-soluble proteins in the present invention has a meaning corresponding to "solubility" of soluble proteins in the field of protein.

The "water-insolubility" of the water-insoluble silver
5 conjugated protein in the present invention means that the protein is hardly dissolved in water or is not dissolved in water essentially, in contrast to the above-mentioned "solubility."

The "whey protein" is contained in a large amount in whey produced as a by-product during production of cheese and
10 essentially contains cystine and the active thiol groups in a relatively large amount. It is possible to obtain a protein containing more active thiol groups by alkali-lysis, enzymatic lysis or treatment with a reducing agent of the whey protein.

The whey protein, which contains water-soluble
15 proteins such as α -lactalbumin and β -lactoglobulin, exists richly in whey produced to be a by-product during the production of cheese and can be industrially purchased.

A commercially available whey protein, for example, is Sunlacto N-5 (tradename) produced by Taiyo Kagaku Co., Ltd.,
20 Japan. The content of the active thiol groups therein is about $50 \mu \text{mol/g}$.

The whey protein commonly contains reduction sugar like lactose and minerals which are derived from a raw material. Since silver ions may be separated as a metal owing to the
25 presence of lactose, lactose is preferably removed before the

whey protein is contacted to silver ions. Lactose may be removed by dissolving the whey protein in deionized water and dialyzing the resulting solution against deionized water.

As a protein containing a lot of intramolecular cystine,
5 may be mentioned scleroprotein keratin forming hair, wool and feathers. However, it is difficult to treat hair and feathers as industrial materials as in the present invention. Wool has a large cystine content, but is disadvantageous for the present invention from the viewpoint of costs.

10 Eggshell membrane protein is a material having a cystine content comparable to that of wool protein, and a hydrolysate or a water-solubilized product thereof is suitably used as a protein of the present invention.

The "eggshell membrane protein" is a water-insoluble
15 protein forming inner membrane of eggshells of birds. The eggshell membrane used in the present invention is preferably made from materials such as chicken eggs or quail eggs which are mass-consumed in the food industry and the like.

This water-insoluble eggshell membrane protein can be
20 made into a water-soluble hydrolysate or a water-solubilized product containing a specific amount of the active thiol groups by subjecting the protein to alkali-lytic, enzymatically lytic or reducing treatment. That is, the treatment is for cleaving a disulfide bond of the eggshell membrane protein to form the
25 active thiol group. It is possible to adjust the thiol group

content in the protein by selecting treatment conditions.

Now the collection of eggshell membrane from eggs and the hydrolysis and water-solubilization of the eggshell membrane are described more particularly.

5 **(Separation of Eggshell Membrane)**

An egg consists of an eggshell, eggshell membrane, albumen and yolk sequentially from the outside. The eggshell in the outermost layer closely contacts the eggshell membrane. In order to collect the eggshell membrane, typically, the eggshell
10 and eggshell membrane are first separated from the albumen and the yolk by breaking an egg. Then the eggshell membrane is peeled off the eggshell, for example, with use of tweezers.

In order to collect a large amount of eggshell membrane industrially, the eggshell and eggshell membrane closely
15 contacting each other are treated with an acid (e.g., a hydrochloric acid of about 10 % concentration) to dissolve calcium carbonate which is a major component of the eggshell. Calcium carbonate is then discarded by filtration.

For effective treatment with the acid, the eggshell and
20 eggshell membrane closely contacting each other are preferably ground mechanically beforehand. Further, it is more preferably to grade the resulting ground product, classify it according to difference in specific gravity and treat it with the acid (see Japanese Unexamined Patent Publication No. HEI
25 3(1991)-45264).

The eggshell membrane separated and collected by the method described in this publication is applied as a seasoning called "Ransho (egg source)" in which the eggshell membrane is decomposed into amino acid, dipeptide or tripeptide and as an ingredient in a cosmetic for activating epidermal cells or fibroblasts as a water-soluble protein.

Next, the eggshell membrane obtained by the above-described method is subjected to the alkali-lysis, enzymatic lysis or reducing treatment as mentioned above to form a water-soluble hydrolysate or a water-solubilized material containing a desired content of the activated thiol groups. Among these treatments, the alkali-lysis is industrially preferable.

(Alkali-lysis)

The eggshell membrane is treated in an about 1 to 30 % aqueous solution (water or an aqueous solution of ethanol concentration of 40 %) of a hydroxide of an alkali metal (e.g., sodium hydroxide or potassium hydroxide). The concentration of the hydroxide of the alkali metal is selected as appropriate depending upon the amount of the eggshell membrane, the treatment temperature and the like. For example, if the amount of the eggshell membrane is about 50 g, the eggshell membrane is treated with 1000 mL of an aqueous solution of the hydroxide of the alkali metal adjusted to 1 N.

The eggshell membrane to which the aqueous solution of the hydroxide of the alkali metal is added is mixed and stirred

for promoting the alkali-lysis. The treatment temperature is about 40 to 80 °C, and the treatment time is about 3 to 24 hours.

The aqueous solution after the treatment is filtered and the resulting filtrate is dialyzed against deionized water, to give an hydrolysate of the eggshell membrane protein.

(Enzymatic lysis)

An hydrolysate is obtained by treating the eggshell membrane with a proteolytic enzyme.

As proteolytic enzymes, may be mentioned plant-origin proteolytic enzymes such as papain and bromelain and animal-origin proteolytic enzymes such as pancreatin, rennin, trypsin, chymotrypsin and pepsin, which are suitably used.

This treatment is carried out in a liquid in which the eggshell membrane is dispersed in water. The treatment temperature and pH are not particularly limited and may be the optimal temperature and pH of the enzyme used. For example, if pancreatin is used, the temperature is suitably 35 to 50 °C, and the pH is suitably about 6 to 8.

The solution after the treatment is filtered and the resulting filtrate is dialyzed against deionized water, to give an hydrolysate of the eggshell membrane protein.

(Treatment with Reducing Agent)

A water-solubilized material can be prepared by treating the eggshell membrane with an reducing agent. By this method, the disulfide bond in the eggshell membrane is

reduced with a reducing agent such as sodium sulfide,
thioglycolic acid, β -thiopropionic acid or an alkali salt thereof,
2-mercaptoethanol or the like. The amount of the reducing
agent used depends on the kind of the agent, but for example, if
5 β -thiopropionic acid is used, the amount of an aqueous β -
thiopropionic acid solution adjusted to 5N is about 2,000 mL
with respect to 100g of the eggshell membrane.

This treatment is carried out in water phase suspending
the eggshell membrane. If β -thiopropionic acid is used as a
10 reducing agent, the treatment temperature is suitably 60 to 80°C
and the treatment time is suitably about 5 hours.

The solution after the treatment is filtered and the
resulting filtrate is dialyzed against deionized water, to give an
hydrolysate of the eggshell membrane protein.

15 The aforesaid whey protein as it is may be used as a
water-soluble protein, but may be used as a hydrolysate or a
water-solubilized material thereof obtained by the above-
described alkali-lysis, enzymatic lysis or treatment with the
reducing agent.

20 The "water-insoluble silver conjugated protein" of the
present invention may be obtained by contacting a water-soluble
protein having an active thiol group content of 0.1 to 200 μ
mol/g with a silver salt in water.

The silver salt is not particularly limited so long as it
25 releases silver ions in water to allow easy binding of silver to

the protein. More particularly, may be mentioned inorganic acid salts such as silver nitrate, silver nitrite, silver sulfate and silver chloride, organic acid salts such as silver acetate, silver lactate and silver oxalate, complex salts such as diamine silver nitrate and diamine silver sulfate, and the like. Silver nitrate and silver acetate are particularly preferable from the viewpoint of solubility to water.

The protein may be contacted to the silver salt in water by use of a known process of mixing/stirring, oscillation or the like, among which mixing/stirring is industrially preferable.

Particular processes using stirring are:

- ① a process of mixing and stirring the protein and the silver salt in water at once;
- ② a process of adding a silver salt dissolved in water little by little into the protein in water under stirring to raise the silver ion concentration in water;
- ③ a process of adding a finely ground silver salt little by little into the protein in water under stirring to raise the silver ion concentration in water;
- ④ a process of adding an aqueous solution of the protein little by little into an aqueous silver salt solution under stirring, finally to keep constant the silver ion concentration in water; and the like.

Among these processes, process ② is particularly preferred because the silver conjugated protein can be obtained

with good reproducibility in a high yield.

The proportion of the silver salt to the protein is preferably about 0.2 to 3 g of the silver salt with respect to 1 g of the protein, although it depends upon the contact conditions.

5 More particularly, it is preferable to use about 1,000 mL of an aqueous silver salt solution having a concentration of about 5 to 250 mM with respect to 1,000 mL of an aqueous protein solution having a concentration of 1 to 20 mg/mL. However, the liquid amount ratio of the aqueous silver salt solution to the aqueous
10 protein solution is not particularly limited so long as a conjugated protein having a high silver content is effectively obtained.

Further, the conditions for contacting the protein to the silver salt may be such that the protein and the silver are
15 uniformly mixed and a conjugated protein having a high silver content is obtained effectively. For example, in the case of contact by stirring, the treatment temperature is suitably about 0 to 70°C, and the treatment time is suitably within 24 hours.

The mixture solution after reaction is filtered and the
20 residue is washed with deionized water, ethanol and the like and dried to give a silver conjugated protein.

The silver content in the resulting conjugated protein can be determined, for example, by quantifying silver eluted using a 3 % nitric acid.

25 According to the present invention, there are provided

an antibacterial/antifungal agent containing the above-described silver conjugated protein as an active component and an antibacterial/antifungal paper treated with the antibacterial/antifungal agent.

5 Medical apparatuses and instruments, stationery, textiles, paper products, daily-use goods, bath goods and the like can exhibit the antibacterial/antifungal activity by being provided with the antibacterial/antifungal agent of the present invention by know processes. Among these object articles, the
10 paper products, in particular, can exhibit excellent antibacterial/antifungal activity.

In the present invention, "paper" means not only sheet-form materials obtained by dispersing cellulose fibers from plants in water, followed by wet papermaking, but also
15 sheet-form materials such as synthetic paper having fiber-entangling structure and properties similar to paper. More particularly, may be mentioned recycled paper from newspaper and board paper, printing paper, copy paper and liner paper.

As processes for treating paper with the antibacterial
20 /antifungal agent of the present invention, may be mentioned a process of coating the surface of paper with the silver conjugated protein dispersed in a suitable amount of water or an aqueous medium, a process of impregnating paper with the silver conjugated protein dispersed in a suitable amount of water or an
25 aqueous medium by immersion or spray (shower) and a process

of making paper from pulp slurry mixed with the silver conjugated protein. The process of coating is particularly preferred because an existing apparatus for papermaking can be converted.

5 In the case where paper is coated with the antibacterial /antifungal agent of the present invention, a preferable coating weight varies depending upon the kind of paper, its use and/or the like, but typically, is 0.01 to 10 g, more preferably 0.1 to 5 g, per square meter. If the coating weight is smaller than 0.01 g
10 per square meter, it is not preferable because a sufficient antibacterial/antifungal effect cannot be obtained. If the coating weight is larger than 10 g per square meter, it is not preferable because a further antibacterial/antifungal effect cannot be expected and the costs rise.

15

Examples

The present invention is now described with preparation examples and test examples, but these preparation and test examples should not be construed to limit the present
20 invention.

Preparation Example 1 (Preparation of hydrolysate of eggshell membrane protein by alkali-lysis)

A 500 mL round flask was charged with a water-
25 containing weight of 74.36 g (a dry weight of 10.81 g) of eggshell

membrane, 130 mL of a 2N NaOH aqueous solution and 86 mL of ethanol, which were stirred at 70°C for 64 hours. Then the mixture solution was filtered and the resulting filtrate was dialyzed against deionized water to give 188 mL of an aqueous solution of a hydrolysate of eggshell membrane protein (referred to as "solubilized protein A" hereinafter).

The protein concentration in the solubilized protein A was determined by the Lowry method, and the active thiol group content in the solubilized protein A was calculated from the protein concentration according to the aforesaid "Quantitative Determination of SH group."

The obtained results are shown below. A protein concentration determined by the Burette method and absorbance (280 nm) are also shown for reference. Numerals in parentheses represent converted total-amount values (total absorbance for the absorbance).

| | |
|----------------------------|--------------------------------|
| Liquid amount | 188 mL |
| Active thiol group content | 17 μ mol/l (3.2 μ mol) |
| Protein concentration | |
| by Lowry Method | 3.4 mg/mL (639.2 mg) |
| by Burette Method | 3.8 mg/mL (714.4 mg) |
| absorbance | 6.2 (1165.6) |

The active thiol group content in the solubilized protein A obtained from the above-mentioned values is 4.5 to 5.0 mol/g.

Preparation Example 2 (Preparation of hydrolysate of eggshell membrane protein by alkali-lysis)

A 500 mL round flask was charged with a water-containing weight of 79 g (a dry weight of 11.46 g) of eggshell membrane, 137 mL of a 2N NaOH aqueous solution and 92 mL of ethanol, which were stirred at 70°C for 96 hours. Then the mixture solution was filtered and the resulting filtrate was dialyzed against deionized water to give 640 mL of an aqueous solution of a hydrolysate of eggshell membrane protein (referred to as "solubilized protein B" hereinafter).

As in Preparation Example 1, the protein concentration in the solubilized protein B was determined and the active thiol group content in the solubilized protein B was calculated from the protein concentration. The obtained results are shown below.

| | |
|----------------------------|---------------------------------|
| Liquid amount | 640 mL |
| Active thiol group content | 55 μ mol/l (35.2 μ mol) |
| Protein concentration | |
| by Lowry Method | 8.0 mg/mL (5120 mg) |
| by Burette Method | 7.2 mg/mL (4608 mg) |
| absorbance | 15.99 (10231) |

The active thiol group content in the solubilized protein B obtained from the above-mentioned values is 6.9 to 7.6 mol/g.

Preparation Example 3 (Preparation of silver conjugated protein

using solubilized protein A)

A 100 mL beaker was charged with 20 mL of the solubilized protein A, to which 20 mL of a 10 mM aqueous solution of silver nitrate were added dropwise under stirring in about 20 minutes. After addition, the mixture solution was stirred for an hour. The resulting mixture solution was allowed to stand overnight and then filtered.

The residue separated by filtration was washed twice with 20 mL of deionized water, then with a small amount of ethanol and was dried to give 59.2 mg of a silver conjugated protein ①.

The silver content in the silver conjugated protein ① was determined by quantifying eluted silver using a 3 % nitric acid. The silver content was 4.75 wt%.

Also the silver ion concentration in the filtrate was determined, and the silver content in the silver conjugated protein ① was calculated from the obtained concentration to be 8.8 wt%. This shows that few silver was released from the silver conjugated protein ① by washing after the filtration, as compared with conventional protein-based antibacterial agents (see Comparative Preparation Examples 1 and 2).

Preparation Example 4 (Preparation of silver conjugated protein using solubilized protein B)

A silver conjugated protein ②, 118.5 mg, was obtained

in the same manner as in Preparation Example 3 except that the solubilized protein B was used instead of the solubilized protein A.

The silver content in the silver conjugated protein ② was 7.0 wt%.

Also the silver ion concentration in the filtrate was determined, and the silver content in the silver conjugated protein ② was calculated from the obtained concentration to be 10.8 wt%. This shows that few silver was released from the silver conjugated protein ② by washing after the filtration, as in Preparation Example 3.

Preparation Example 5 (Preparation of silver conjugated protein using whey protein)

In a 300 mL beaker, 2 g of whey protein (tradename: Sunlacto N-5 produced by Taiyo Kagaku Co., Ltd., with a protein content of 72 % and an active thiol group content of 47 μ mol/g) were dissolved in 200 mL of deionized water. To this mixture solution, 200 mL of a 50 mM aqueous solution of silver nitrate were added and stirred for an hour. The resulting mixture solution was allowed to stand overnight and filtered (using a filter paper No.2).

The residue separated by filtration was washed twice with 100 mL of deionized water and dried to give 1646 mg of a silver conjugated protein ③ (a yield of 115 % with respect to the

protein used).

The silver content in the silver conjugated protein ③ was determined by quantifying eluted silver using a 3 % nitric acid. The silver content was 4.25 wt%.

5 Also the silver ion concentration in the filtrate was determined, and the silver content in the silver conjugated protein ③ was calculated from the obtained concentration to be 4.86 wt%. This shows that few silver was released from the silver conjugated protein ③ by washing after the filtration, as
10 in Preparation Example 3.

Preparation Example 6 (Preparation of silver conjugated protein using whey protein)

In a 300 mL beaker, 2 g of whey protein (tradename:
15 Sunlacto N-5 produced by Taiyo Kagaku Co., Ltd., with a protein content of 72 % and an active thiol group content of 47 μ mol/g) were dissolved in 180 mL of deionized water. This mixture liquid was dialyzed against deionized water to remove lactose.

20 20 mL of a 10 mM aqueous solution of silver nitrate were added to 20 mL of the collected dialysate and the mixture was stirred for an hour. The resulting mixture solution was allowed to stand overnight and filtered (using a filter paper No.2).

The residue separated by filtration was washed twice
25 with 50 mL of deionized water and dried to give 154.8 mg of a

silver conjugated protein ④.

The silver content in the silver conjugated protein ④ was determined by quantifying eluted silver using a 3 % nitric acid. The silver content was 7.80 wt%.

5

Preparation Example 7 (Preparation of silver conjugated protein using whey protein)

In a 5 L beaker, 30 g of whey protein (tradename: ALACEN 895 produced by New Zealand Milk Product, with a
10 protein content of 86.5 % and an active thiol group content of 34.5 μ mol/g) were dissolved in 3 L of deionized water.

To the aqueous solution of the whey protein, was added an aqueous solution of 4.574 g of silver nitrate which had been dissolved in about 300 mL of deionized water in a separate
15 beaker. The mixture was stirred for an hour. The resulting mixture solution was allowed to stand overnight and spray-dried.

The spray drying was performed with an L-8 type spray drier (produced by Okawahara Kakoki Kabushiki Kaisha) at a
20 flow rate of 2 L/ hour under the conditions of a hot-air inlet temperature of 180°C and an air outlet temperature of 95°C, to give a silver conjugated protein ⑤.

After the obtained silver conjugated protein ⑤ was decomposed, the silver content was determined by the
25 ammonium thiocyanate titration method described in Japanese

Pharmacopoeia. The silver content was 9.30 wt%.

Preparation Example 8 (Preparation of silver conjugated protein using whey protein)

5 In a 5 L beaker, 25 g of whey protein (tradename: ALACEN 895 produced by New Zealand Milk Product, with a protein content of 86.5 % and an active thiol group content of 34.5 μ mol/g) were dissolved in 2.5 L of deionized water.

To the aqueous solution of the whey protein, was added
10 an aqueous solution of 21.24 g of silver nitrate which had been dissolved in about 2.5 L of deionized water in a separate beaker. The mixture was stirred for an hour. The resulting mixture solution was allowed to stand overnight and spray-dried in the same manner as in Preparation Example 3, to give a silver
15 conjugated protein ⑥.

After the obtained silver conjugated protein ⑥ was decomposed, the silver content was determined by the ammonium thiocyanate titration method described in Japanese Pharmacopoeia. The silver content was 31.15 wt%.

20

Preparation Example 9 (Preparation of silver conjugated protein using eggshell membrane)

About 40 g of wet weight of raw eggshell membrane (having an active thiol group content of 5 μ mol/g) separated
25 from raw eggs are immersed in 800 mL of a 50 mM aqueous

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solution of silver nitrate, dispersed by a mixer and allowed to stand overnight. Thereafter, eggshell membrane was collected by filtration and washed with 800 mL of deionized water. The filtering and washing were repeated three times. The eggshell membrane after washing was dispersed in 400 mL of deionized water, and wet-ground by a rotary ball mill. Grinding was continuously performed until the average particle diameter in the slurry became smaller than 20 to 30 microns. Thereafter, the slurry liquid was collected, and the ground product was filtered, washed and dried by heat under reduced pressure, to give a silver conjugated protein ⑦.

After the obtained silver conjugated protein ⑦ was decomposed, the silver content was determined by the ammonium thiocyanate titration method described in Japanese Pharmacopoeia. The silver content was 6.36 wt%.

Comparative Preparation Example 1 (Preparation of silver conjugated protein using mechanically ground eggshell membrane)

In a 50 mL beaker, 2 g of a mechanically ground eggshell membrane ① (tradename : Sunkakumaku P produced by Taiyo Kagaku Co., Ltd., the active thiol group content cannot be determined) was suspended by adding 10 ml of a 0.2 % aqueous solution of silver nitrate. This suspension was stirred at room temperature for 10 minutes and filtered (using a filter

paper No.2).

The residue separated by filtration was washed three times with 50 mL of deionized water and dried to give 1904 mg of a silver-adsorbing eggshell membrane powder ①.

5 The silver content in the silver-adsorbing eggshell membrane powder ① was determined by quantifying eluted silver using a 3 % nitric acid. The silver content was 0.059 wt%.

Also the silver ion concentration in the filtrate was
10 determined, and the silver content in the silver-adsorbing eggshell membrane powder ① was calculated from the obtained concentration to be 0.68 wt%. This shows that silver was released from the silver-adsorbing eggshell membrane powder ① by washing after the filtration.

15

Comparative Preparation Example 2 (Preparation of silver conjugated protein using mechanically ground eggshell membrane)

1928 mg of a silver-adsorbing eggshell membrane
20 powder ② (the active thiol group content was not able to be determined) was obtained in the same manner as in Comparative Preparation Example 1 except that a mechanically ground eggshell membrane ② obtained by grinding with a stone mill and by air-grinding/gradation was used instead of the
25 mechanically ground eggshell membrane ①.

The silver content in the silver-adsorbing eggshell membrane powder ② was 0.026 wt%.

Also the silver ion concentration in the filtrate was determined, and the silver content in the silver-adsorbing eggshell membrane powder ② was calculated from the obtained concentration to be 0.67 wt%. This shows that silver was released from the silver-adsorbing eggshell membrane powder ② by washing after the filtration.

10. Test Example 1 (Test of silver conjugated proteins on their antibacterial power against Staphylococcus aureus)

The silver conjugated protein ① and the silver conjugated protein ③ were tested on their antibacterial power against *Staphylococcus aureus* under the following conditions.

15 Known silver-containing antibacterial agents were also tested.

The known silver-containing antibacterial agents were as follows:

Zeomic [tradename (registered trademark), produced by
SINANEN ZEOMIC CO., LTD., Japan, containing about 2.5 wt%
silver]

Novaron [tradename (registered trademark, produced by Toagosei Co., Ltd., Japan)]

The obtained results are shown in Table 1. The numerical values in the table represent viable cell counts, each of which is the average of two samples. Accordingly, two viable

cell count data are shown in a "not added" row. In the following tests, likewise, the viable cell count represents the average of viable cell counts in two samples.

Determination method : in accordance with the
5 determination of the minimum bactericidal concentration (MBC)
of Antibacterial Evaluation Tests For Inorganic Antibacterial
Agents Such As Silver II (1995 edition, edited by the Society for
the Study of Inorganic Antibacterial Agents Such As Silver)

Test bacteria : Staphylococcus aureus IFO 12732

10 Media used : SCD medium

SCD agar medium.

The minimum bactericidal concentrations (MBC values)
of the silver conjugated protein ① and the silver conjugated
protein ③ against Staphylococcus aureus were 3.13 ppm and
15 6.25 ppm, respectively.

Table 1

| Concentration of agent added (ppm) | Silver conjugated protein ① | Silver conjugated protein ③ | Zeomic | Novaron | Concentration of Ag in silver nitrate (ppm) | Silver nitrate |
|------------------------------------|--|-----------------------------|--------|----------|---|----------------|
| Not added | 8.19×10^5 6.90×10^5 | ditto | ditto | ditto | ditto | ditto |
| 3200 | 0 | 0 | 0 | ∞ | 6.25 | 0 |
| 1600 | 0 | 0 | 0 | ∞ | 3.13 | 0 |
| 800 | 0 | 0 | 0 | ∞ | 1.56 | 0 |
| 400 | 0 | 0 | 0 | ∞ | 0.78 | 1 |
| 200 | 0 | 0 | 0 | ∞ | 0.39 | ∞ |
| 100 | 0 | 0 | 0 | ∞ | 0.2 | ∞ |
| 50 | 0 | 0 | 0 | ∞ | 0.1 | ∞ |
| 25 | 0 | 0 | 0 | ∞ | 0.05 | ∞ |
| 12.5 | 0 | 0 | 296 | ∞ | 0.025 | ∞ |
| 6.25 | 0 | 132 | 32 | ∞ | 0.013 | ∞ |

* Test bacteria : Staphylococcus aureus

* It is judged that bacteria has not grown where the viable cell count is not more than 5.

- 5 * The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

10 Test Example 2 (Test of silver conjugated proteins on their antibacterial power against Escherichia coli)

The silver conjugated protein ①, the silver conjugated protein ② and the silver conjugated protein ③ were tested on their antibacterial power against Escherichia coli under the following conditions. As in Test Example 1, comparative test was also carried out with regard to the known silver-containing antibacterial agents. The agents were added in concentrations within the range of 3.13 to 800.00 ppm.

The obtained results are shown in Table 2. The numerical values in the table represent viable cell counts.

Determination method : in accordance with the determination of the minimum bactericidal concentration (MBC) of Antibacterial Evaluation Tests For Inorganic Antibacterial Agents Such As Silver II (1995 edition, edited by the Society for the Study of Inorganic Antibacterial Agents Such As Silver)

Test bacterial : Escherichia coli IFO 3972

Media used : Normal bouillon medium (NB medium)

Normal bouillon agar medium (NA medium).

The minimum bactericidal concentrations (MBC values) of the silver conjugated protein ①, the silver conjugated protein ② and the silver conjugated protein ③ against Escherichia coli were less than 1.65 ppm, 1.65 ppm and 6.25 ppm, respectively.

That of Zeomic was 6.25 ppm, and that of Novaron was 50 ppm.

Table 2

| Concentration of agent added (ppm) | Silver conjugated protein ① | Silver conjugated protein ② | Silver conjugated protein ③ | Zeomic | Novaron | Concentration of Ag in silver nitrate (ppm) | Silver nitrate |
|------------------------------------|--|-----------------------------|-----------------------------|----------|----------|---|----------------|
| Not added | 1.72×10^7 1.70×10^7 | ditto | ditto | ditto | ditto | ditto | ditto |
| 800.00 | 0 | 0 | 0 | 0 | 0 | 6.25 | 0 |
| 400.00 | 0 | 0 | 0 | 0 | 0 | 3.13 | 0 |
| 200.00 | 0 | 0 | 0 | 0 | 0 | 1.56 | 0 |
| 100.00 | 0 | 0 | 0 | 0 | 0 | 0.78 | 0 |
| 50.00 | 0 | 0 | 0 | 0 | 13 | 0.39 | 0 |
| 25.00 | 0 | 0 | 0 | 0 | 16 | 0.2 | 0 |
| 12.50 | 0 | 0 | 0 | 0 | ∞ | 0.1 | ∞ |
| 6.25 | 0 | 0 | 51 | 44 | ∞ | 0.05 | ∞ |
| 3.13 | 0 | 2 | 203 | ∞ | ∞ | | |

* The final active concentration of an agent is half the concentration shown above.

* It is judged that bacteria has not grown where the viable cell count is not more than 5.

Test Example 3 (Test of silver conjugated proteins on their antibacterial power against Escherichia coli)

The silver conjugated protein ⑤, the silver conjugated protein ⑥ and the silver conjugated protein ⑦ were tested on their antibacterial power against Escherichia coli under the same conditions as in Test Example 2. The agents were added in concentrations within the range of 0.049 to 25.000 ppm.

The obtained results are shown in Table 3. The

numerical values in the table represent viable cell counts.

The minimum bactericidal concentrations (MBC values) of the silver conjugated protein ⑤, the silver conjugated protein ⑥ and the silver conjugated protein ⑦ against *Escherichia coli* were 0.39 ppm, 0.098 ppm and 0.78 ppm, respectively.

Table 3

| Concentration of agent added (ppm) | Silver conjugated protein ⑤ | Silver conjugated protein ⑥ | Silver conjugated protein ⑦ | Concentration of Ag in silver nitrate (ppm) | Silver nitrate |
|------------------------------------|--|-----------------------------|-----------------------------|---|----------------|
| Not added | 1.30×10^7 1.70×10^7 | ditto | ditto | ditto | ditto |
| 25.000 | 0 | 0 | 0 | 0.780 | 0 |
| 12.500 | 0 | 0 | 0 | 0.390 | 0 |
| 6.250 | 0 | 0 | 0 | 0.200 | 0 |
| 3.130 | 0 | 0 | 0 | 0.100 | 0 |
| 1.560 | 0 | 0 | 0 | 0.050 | 0 |
| 0.781 | 0 | 0 | 23 | 0.025 | 17 |
| 0.391 | 42 | 0 | 781 | 0.013 | 1611 |
| 0.196 | 132 | 0 | ∞ | | |
| 0.098 | 1354 | 17 | ∞ | | |
| 0.049 | ∞ | 73 | ∞ | | |

* The final active concentration of an agent is half the concentration shown above.

* It is judged that bacteria has not grown where the viable cell count is not more than 5.

Test Example 4 (Test of silver conjugated proteins on their antibacterial power against *Staphylococcus aureus*)

The mechanically ground eggshell membrane ①, the silver-adsorbing eggshell membrane powder ①, the mechanically ground eggshell membrane ② and the silver-adsorbing eggshell membrane powder ② were tested on their antibacterial power against Staphylococcus aureus under the following conditions.

Determination method : in accordance with the determination of the minimum bactericidal concentration (MBC) of Antibacterial Evaluation Tests For Inorganic Antibacterial Agents Such As Silver II (1995 edition, edited by the Society for the Study of Inorganic Antibacterial Agents Such As Silver)

Test bacterial : Staphylococcus aureus IFO 12732

Media used : SCD medium

SCD agar medium.

The antibacterial power test was carried out with varying the concentrations of the agents within the range of 1.56 ppm to 400 ppm. The antibacterial power was not recognized with any of the mechanically ground eggshell membrane ①, the silver-adsorbing eggshell membrane powder ①, the mechanically ground eggshell membrane ② and the silver-adsorbing eggshell membrane powder ②.

Preparation Example 10 (Effect of the concentration of silver nitrate on the formation of silver conjugated protein)

Silver conjugated proteins were prepared in the same

manner as in Preparation Example 3 except that the concentration of the aqueous solution of silver nitrate was varied to 0.1 mM, 0.5 mM, 2 mM, 5 mM, 10 mM and 50 mM.

The silver ion concentration in the filtrates after separating the silver conjugated proteins by filtration was determined, and the silver content in the silver conjugated proteins was calculated from the obtained values.

The yield was also calculated from the amount of protein, 68 mg, in the solubilized protein A (having a protein concentration of 3.4 mg/mL by the Lowry method, 20 mL) and the obtained amount of the silver conjugated proteins.

The obtained results are shown in Fig. 1.

Preparation Example 11 (Effect of the concentration of silver nitrate on the formation of silver conjugated protein)

Silver conjugated proteins were prepared in the same manner as in Preparation Example 4 except that the concentration of the aqueous solution of silver nitrate was varied to 0.1 mM, 0.5 mM, 2 mM, 5 mM, 10 mM and 50 mM.

The silver ion concentration in the filtrates after separating the silver conjugated proteins by filtration was determined, and the silver content in the silver conjugated proteins was calculated from the obtained values.

The yield was also calculated from the amount of protein, 160 mg, in the solubilized protein B (having a protein

concentration of 8.0 mg/mL by the Lowry method, 20 mL) and the obtained amount of the silver conjugated proteins.

The obtained results are shown in Fig. 2.

It is understood from Fig. 1 and Fig. 2 that silver
5 conjugated proteins having high silver contents are obtained in high yields.

Test Example 5 (Comparative test of antibacterial power of silver
conjugated protein and known silver-containing antibacterial
10 agents)

Comparative test of the antibacterial power of the silver
conjugated protein ② (having a silver content of 7.0 wt%) and
known silver-containing antibacterial agents against
Staphylococcus aureus was carried out under the same
15 conditions as in Test Example 1.

The known silver-containing antibacterial agents were
aforesaid Zeomic and Novaron, and silver nitrate was also tested
for reference.

The obtained results are shown in Table 4. The
20 numerical values in the table represent viable cell counts.

Table 4

| Concentration of agent added (ppm) | Silver conjugated protein ② | Zeomic | Novaron | Concentration of Ag in silver nitrate (ppm) | Silver nitrate |
|------------------------------------|--|----------|----------|---|----------------|
| Not added | 2.65×10^6 3.76×10^6 | ditto | ditto | ditto | ditto |
| 3200 | 0 | 0 | ∞ | 6.25 | 0 |
| 1600 | 0 | 0 | ∞ | 3.13 | 0 |
| 800 | 0 | 0 | ∞ | 1.56 | 0 |
| 400 | 0 | 3 | ∞ | 0.78 | 1 |
| 200 | 0 | 1 | ∞ | 0.39 | ∞ |
| 100 | 0 | 5 | ∞ | 0.2 | ∞ |
| 50 | 0 | 16 | ∞ | 0.1 | ∞ |
| 25 | 0 | ∞ | ∞ | 0.05 | ∞ |
| 12.5 | 0 | ∞ | ∞ | 0.025 | ∞ |
| 6.25 | 0 | ∞ | ∞ | 0.013 | ∞ |

* Test bacteria : Staphylococcus aureus

* It is judged that bacteria has not grown where the viable cell count is not more than 5.

5 * The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

Test Example 6 (Comparative test of antibacterial power of silver conjugated protein and known silver antibacterial agents)

Comparative test of the antibacterial power of the silver

conjugated protein ③ (having a silver content of 4.25 wt%) and known silver-containing antibacterial agents against *Staphylococcus aureus* was carried out under the same conditions as in Test Example 1.

5 The known silver-containing antibacterial agents were aforesaid Zeomic and Novaron, and silver nitrate was also tested for reference.

The obtained results are shown in Table 5. The numerical values in the table represent viable cell counts.

10

Table 5

| Concentration of agent added (ppm) | Silver conjugated protein ③ | Zeomic | Novaron | Concentration of Ag in silver nitrate (ppm) | Silver nitrate |
|------------------------------------|--|----------|----------|---|----------------|
| Not added | 2.65×10^6 3.76×10^6 | ditto | Ditto | ditto | ditto |
| 3200 | 0 | 0 | ∞ | 6.25 | 0 |
| 1600 | 0 | 0 | ∞ | 3.13 | 0 |
| 800 | 0 | 0 | ∞ | 1.56 | 0 |
| 400 | 0 | 3 | ∞ | 0.78 | 1 |
| 200 | 0 | 1 | ∞ | 0.39 | ∞ |
| 100 | 0 | 5 | ∞ | 0.2 | ∞ |
| 50 | 0 | 16 | ∞ | 0.1 | ∞ |
| 25 | 1 | ∞ | ∞ | 0.05 | ∞ |
| 12.5 | 20 | ∞ | ∞ | 0.025 | ∞ |
| 6.25 | ∞ | ∞ | ∞ | 0.013 | ∞ |

* Test bacteria : *Staphylococcus aureus*

* It is judged that bacteria has not grown where the viable cell count is not more than 5.

* The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

Test Example 7 (Comparative test of antibacterial power of silver conjugated protein and known silver-containing antibacterial agent)

10 Comparative test of the antibacterial power of the silver conjugated protein ② (having a silver content of 7.0 wt%) and a known silver-containing antibacterial agent against *Staphylococcus aureus* was carried out under the same conditions as in Test Example 1.

15 The known silver-containing antibacterial agent was as follows, and silver nitrate was also tested for reference.

BIKAHM AK-LS (produced by Otsuka Chemical Co., Ltd., Japan, having a silver content of 15 wt%).

The obtained results are shown in Table 6. The
20 numerical values in the table represent viable cell counts.

| Concentration of agent added (ppm) | Silver conjugated protein ② | BIKAHM AK-LS (Otsuka Chemical Co., Ltd. | Concentration of Ag in silver nitrate (ppm) | Silver Nitrate |
|------------------------------------|-----------------------------|---|---|----------------|
| Not added | 3.50×10^6 | ditto | ditto | ditto |
| 200.00 | 0 | ∞ | 12.50 | 0 |
| 100.00 | 0 | ∞ | 6.25 | 1 |
| 50.00 | 0 | ∞ | 3.13 | 1 |
| 25.00 | 0 | ∞ | 1.56 | 1 |
| 12.50 | 0 | ∞ | 0.78 | 133 |
| 6.25 | 4 | ∞ | 0.39 | ∞ |
| 3.13 | 14695 | ∞ | 0.20 | ∞ |
| 1.56 | ∞ | ∞ | 0.10 | ∞ |
| 0.78 | ∞ | ∞ | | |

* Test bacteria : Staphylococcus aureus

* It is judged that bacteria has not grown where the viable cell count is not more than 5.

- 5 * The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

10 Test Example 8 (Comparative test of antibacterial power of silver conjugated protein and official silver protein)

Comparative test of the antibacterial power of the silver conjugated protein ② (having a silver content of 7.0 wt%) and

Table 7

| Concentration of agent added (ppm) | Silver conjugated protein ② | Official Protein Silver (MARUISHI Pharmaceutical Co. Ltd.) | Concentration of Ag in silver nitrate (ppm) | Silver Nitrate |
|------------------------------------|-----------------------------|--|---|----------------|
| Not added | 3.50×10^6 | ditto | ditto | ditto |
| 200.00 | 0 | 0 | 12.50 | 0 |
| 100.00 | 0 | 0 | 6.25 | 1 |
| 50.00 | 0 | 0 | 3.13 | 1 |
| 25.00 | 0 | 0 | 1.56 | 1 |
| 12.50 | 0 | 128 | 0.78 | 133 |
| 6.25 | 4 | 170 | 0.39 | ∞ |
| 3.13 | 14695 | ∞ | 0.20 | ∞ |
| 1.56 | ∞ | ∞ | 0.10 | ∞ |
| 0.78 | ∞ | ∞ | | |

* Test bacteria : Staphylococcus aureus

* It is judged that bacteria has not grown where the viable cell count is not more than 5.

5 * The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

10 It is understood from the results shown in Tables 1 to 6 that the silver conjugated proteins of the present invention have more excellent antibacterial/antifungal activity than the known inorganic antibacterial agents containing silver. BIKAHM AK-LS, a known silver-containing antibacterial agent, used in Test

Example 7 whose results are shown in Table 6 is particularly inferior in the antibacterial/antifungal activity although its silver content is as high as 15 wt%. That is considered because silver ions are not released in a sustained manner in an appropriate amount when the antibacterial activity should be exhibited. As clearly understood from the results in Table 7, the antibacterial/antifungal activity of the silver conjugated proteins of the present invention is more excellent than that of the official protein.

Test Example 9 (Comparative test of fungal resistance of silver conjugated proteins and known silver-containing antibacterial agent)

Comparative test of the fungal resistance of the silver conjugated protein ①, the silver conjugated protein ②, the silver conjugated protein ③ and the known silver-containing antibacterial agent [Zeomic (tradename, produced by SHINANEN ZEOMIC CO., LTD.)] was carried out under the following conditions.

Determination method : in accordance with JIS K2911-1981 fungal resistance test method, particularly the following items:

- 3. Preparation for test 3.5 Spore suspension
- 4. General rule of test 4.3.2. Expression of test results
- Table 1 "How to express test results"
- 6. Test of fiber products 6.2.2. Wet method

Fungi : First group : *Aspergillus niger* (FERM S-1)
 Second group : *Penicillium citrinum* (FERM S-5)
 Third group : *Rhizopus stolonifer* (FERM S-7)
 Fourth group : *Cladosporium cladosporioides*
 (FERM S-8)

Fifth group : *Chaetomium globosum* (FERM S-11)

Commercially available copy paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by SASUDA SEIKI) under the condition of a coating thickness of 1 to 10 μ m. Each agent was prepared in the following concentrations. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm \times 5 cm) having an intended coating weight.

The agent of the silver conjugated protein ① was prepared by dispersing 256 mg of the silver conjugated protein ① in 20 mL of deionized water. This agent was used for producing coated test pieces with coating weights of 1g/m², 2g/m² and 4g/m².

The agent of the silver conjugated protein ② was prepared by dispersing 256 mg and 1280 mg of the silver conjugated protein ② each in 20 mL of deionized water. This agent was used for producing coated test pieces with coating weights of 1g/m², 2g/m² and 4g/m².

The agent of the silver conjugated protein ③ was prepared by dispersing 256 mg of the silver conjugated protein

③ in 20 mL of deionized water. This agent was used for producing coated test pieces with coating weights of 1g/m^2 , 2g/m^2 and 4g/m^2 .

Zeomic was dispersed in deionized water to prepare a 1% agent and a 10% agent. These agents were used for producing coated test pieces with coating weights of 1g/m^2 , 2g/m^2 and 4g/m^2 .

The growth of a fungus was observed on test pieces coated with the antibacterial/antifungal agents in varied coating weights (on both faces). Uncoated paper was tested under the same conditions as control for every test.

Figs. 3(a), 3(b) and 3(c) show the growth of a fungus with varying the coating weight of the silver conjugated protein ② to 1g/m^2 , 2g/m^2 and 4g/m^2 .

Figs. 4(a), 4(b) and 4(c) show the growth of a fungus with varying the coating weight of the silver conjugated protein ③ to 1g/m^2 , 2g/m^2 and 4g/m^2 .

Figs. 5(a), 5(b) and 5(c) show the growth of a fungus with varying the coating weight of Zeomic to 1g/m^2 , 2g/m^2 and 4g/m^2 .

The results of the fungal resistance test are also shown in Table 8.

Table 8

| | Silver conjugated protein ② | | | Silver conjugated protein ③ | | | Zeomic | | | Control |
|----------------|--------------------------------|--------------------|--------------------|--------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|------------|
| Coating weight | 1 g/m ² | 2 g/m ² | 5 g/m ² | 1 g/m ² | 2 g/m ² | 4 g/m ² | 1 g/m ² | 2 g/m ² | 10g/m ² | Not coated |
| Judge-ment | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 1 | 3 | 1 |

1 : The area where hyphae grew exceeded one-third of the total area.

2 : The area where hyphae grew did not exceed one-third of the total area.

3 : The growth of hyphae was not observed.

It is understood from the results shown in Table 8 that the silver conjugated proteins ② and ③ exhibit much more excellent fungal resistance than the known silver-containing antibacterial agent.

The silver conjugated proteins ② and ③, if used at an increased coating weight, are observed to change color due to silver. However, from the viewpoint of the fungal resistance, sufficient is a small coating weight such that a change of color does not take place. Therefore, there are no problems with the change of color.

Test Example 10 (Fungal resistance test of silver conjugated protein)

Pieces of paper coated with the silver conjugated protein ③ were tested on their fungal resistance in accordance with JIS K2911-1981 fungal resistance test method.

Fungi used : *Neosartorya fischeri*

Liner paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by YASUDA SEIKI, Japan) under the condition of a coating thickness of 1 μ m or 3 μ m. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm × 5 cm) having a coating weight of 0.14 g/m² and 0.42 g/m².

The obtained results are shown in Fig. 6 and Fig. 7.

Fig. 6 shows the growth of the fungus where the silver

conjugated protein ③ was applied on the liner paper at a coating weight of 0.14g/m².

Fig. 7 shows the growth of the fungus where the silver conjugated protein ③ was applied on the liner paper at a coating weight of 0.42 g/m².

The results of the fungal resistance test are also shown in Table 9.

Table 9

| | Silver conjugated protein ③ | | | Silver conjugated protein ③ | | | Control |
|----------------|-----------------------------|------|------|-----------------------------|------|------|------------|
| Coating weight | 0.14 g/m ² | | | 0.42 g/m ² | | | Not coated |
| Test piece | No.1 | No.2 | No.3 | No.1 | No.2 | No.3 | No.1 |
| Judgement | 3 | 3 | 3 | 3 | 3 | 3 | 1 |

1 : The area where hyphae grew exceeded one-third of the total area.

2 : The area where hyphae grew did not exceed one-third of the total area.

3 : The growth of hyphae was not recognized.

It is understood from Fig. 6, Fig. 7 and the results shown in Table 9, the silver conjugated protein ③ also provides good fungal resistance to liner paper whose both faces are coated at coating weights of 0.14 g/m² and 42 g/m².

Test Example 11 (Fungal resistance test of silver conjugated proteins)

Pieces of paper coated with the silver conjugated proteins ⑤ to ⑦ were tested on their fungal resistance in accordance with JIS K2911-1981 fungal resistance test method.

Liner paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by YASUDA SEIKI) under the condition of a coating thickness of 1 μm or 3 μm. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm × 5 cm) having an intended coating weight.

Fungi used : Spore suspension of *Neosartorya fischeri*

The growth of the fungi was observed with varying the coating weight of the antibacterial/antifungal agents (applied on both faces) on the test pieces. Uncoated paper was tested under the same conditions as control for every test.

The obtained results are shown in Table 10.

Table 10

| | Silver conjugated proteins ⑤ | | | Silver conjugated proteins ⑥ | | | Silver conjugated proteins ⑦ | | | |
|----------------|------------------------------|-----------------------|-----------------------|------------------------------|-----------------------|-----------------------|------------------------------|-----------------------|-----------------------|------------|
| Coating weight | 0.15 g/m ² | 0.45 g/m ² | 0.90 g/m ² | 0.15 g/m ² | 0.45 g/m ² | 0.90 g/m ² | 0.15 g/m ² | 0.45 g/m ² | 0.90 g/m ² | Not Coated |
| 1st judgement | 2 | 2 | 3 | 2 | 3 | 3 | 1 | 2 | 2 | 1 |
| 2nd judgement | 2 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 1 |
| 3rd judgement | 2 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 1 |

1 : The area where hyphae grew exceeded one-third of the total area.

2 : The area where hyphae grew did not exceed one-third of the total area.

3 : The growth of hyphae was not recognized.

It is understood from Table 10 that the test pieces coated with the silver conjugated proteins ⑤ to ⑦ have a judgement of 2 or more and have good fungal resistance while the uncoated test pieces have a judgement of 1. The silver conjugated proteins exhibit a remarkable effect when used particularly at a coating weight of 0.45 g/m² or more.

Test Example 12 (Fungal resistance test of silver conjugated protein)

Pieces of paper coated with the silver conjugated protein ③ were tested on their fungal resistance in accordance with JIS K2911-1981 fungal resistance test method.

Liner paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by YASUDA SEIKI) under the condition of a coating thickness of 1 μm or 3 μm. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm × 5 cm) having an intended coating weight.

Fungi used : the same spore suspension as used in Test Example 9

The obtained results are shown in Fig. 8 and Fig. 9.

Fig. 8 shows the growth of the fungus where the silver conjugated protein ③ was applied on the liner paper at a coating weight of 0.14g/m².

Fig. 9 shows the growth of the fungus where the silver

conjugated protein ③ was applied on the liner paper at a coating weight of 0.42 g/m².

The results of the fungal resistance test are also shown in Table 11

Table 11

| | Silver conjugated protein ③ | | | Silver conjugated protein ③ | | | Control |
|-------------------|--------------------------------|------|------|--------------------------------|------|------|------------|
| Coating weight | 0.14 g/m ² | | | 0.42 g/m ² | | | Not coated |
| Test piece | No.1 | No.2 | No.3 | No.1 | No.2 | No.3 | No.1 |
| Judgement | 2 | 3 | 3 | 3 | 3 | 3 | 1 |

1 : The area where hyphae grew exceeded one-third of the total area.

2 : The area where hyphae grew did not exceed one-third of the total area.

3 : The growth of hyphae was not recognized.

It is understood from Fig. 8, Fig. 9 and the results shown in Table 11 that the silver conjugated protein ③ also provides good fungal resistance to liner paper whose both faces are coated at coating weights of 0.14 g/m² and 42 g/m².

Test Example 13 (Observation of paper coated with silver conjugated proteins by scanning electronic microscope)

The surface of the copy paper coated with the silver conjugated protein ①, the silver conjugated protein ③ and Zeomic, a known silver-containing antibacterial agent, in Test Example 9 was observed by a scanning electronic microscope.

The obtained results are shown in Fig. 10 to Fig. 12.

Fig. 10 shows the state of the surface of the copy paper coated with the silver conjugated protein ①.

Fig. 11 shows the state of the surface of the copy paper coated with the silver conjugated protein ③.

Fig. 12 shows the state of the surface of the copy paper coated with Zeomic.

It is observed in Fig. 10 and Fig. 11 that the silver conjugated proteins ① and ③ adhere to the surface of the paper like a film filling spaces between pulp fibers. On the other hand, it is observed in Fig. 12 that the known silver-containing antibacterial agent Zeomic adheres to the surface of paper as fine particles. However, these particles easily fall away by being touched with fingers.

For this reason, it is understood that inorganic silver antibacterial agent, when used by being applied onto the surface of paper, needs a resin binder (an adhesive or a binder). In contrast, the silver conjugated proteins of the present invention have properties such as a film-forming property, a sticking property and the like which come from the raw material proteins. Therefore, the silver conjugated proteins can be applied to paper without using adhesives or binders.

Test Example 14 (KBB size degree test of paper coated with silver conjugated proteins)

The KBB size degree test was carried out on the copy paper coated with the silver conjugated protein ①, the silver conjugated protein ③ and the known silver-containing antibacterial agent Zeomic in Test Example 9, in accordance with JIS P8122. The test was performed by use of an automatic KBB size degree measuring apparatus produced by YASUDA SEIKI.

The obtained results are shown in Table 12.

Table 12

(uncoated paper) commercially available copy paper : KBB size degree 86.9 seconds in average

| Coating weight of silver conjugated protein ① (g/m ²) | KBB size degree (sec.) | Coating weight of silver conjugated protein ③ (g/m ²) | KBB size degree (sec.) | Coating weight of Zeomic (g/m ²) | KBB size degree (sec.) |
|---|---------------------------|---|---------------------------|---|---------------------------|
| 1 | 56.7 | 1 | 102 | 1 | 115.8 |
| 2 | 1600.0 | 2 | 125 | 2 | 116.4 |
| 5 | 3800.0 | 4 | 162 | 10 | 122.0 |

Note : the coating weight is the sum of the coating weights on both faces

It is understood from the results shown in Table 12 that the copy paper coated with the silver conjugated protein ① and the silver conjugated protein ③ has a larger size degree as the coating weight increases. The silver conjugated protein ①, especially, provides a remarkable increase in the size degree. On the other hand, the copy paper coated with Zeomic has an increase in the size degree, though the increase is small. That is because a lot of Zeomic particles adhere to the surface of the paper and, as the thickness of the particles increases, more time is required for penetration of an electrolytic liquid, which is apparently observed as an increase in the size degree.

Test Example 15 (Test of antibacterial activity of liner paper coated with silver conjugated proteins)

Pieces of paper coated with the silver conjugated proteins ⑤ to ⑦ were tested on their antibacterial activity in accordance with JIS L1902.

For the test, the coated test pieces in Test Example 11 were cut into 18 mm × 18 mm beforehand and used as test pieces. *Staphylococcus aureus* IFO 12732, a bacterium used, was inoculated into a SCD agar medium and cultured at 37 °C for 24 to 48 hours. After culture, a loopful of the resulting culture was collected, inoculated into an L-shaped tube fed with 5 mL of the SCD medium and cultured at 37 °C for 18 hours. The resulting culture was diluted 10 fold with a sterilized

physiological saline and diluted with the SCD medium so that the absorbance at 660 nm was 0.1. Thereby the viable cell count became $1 \text{ to } 2 \times 10^8 / \text{mL}$. The resulting liquid was diluted with a 20-fold dilution liquid of the SCD medium ice-cooled so that the viable cell count became $1 \pm 0.3 \times 10^5 / \text{mL}$, thereby obtaining test bacteria.

Six pieces of uncoated paper and three pieces of coated paper were prepared. The test pieces were put at the bottom of vials autoclaved at 121°C for 15 minutes. Thereafter, 0.2 mL of the prepared test bacteria were inoculated uniformly on several sites of each of the test pieces and cultured at 37°C for 18 hours.

After culture, 20 ml of physiological saline were fed into each vial and stirred (for 5 seconds, 5 times) by a test tube oscillator to wash out remaining bacteria.

One mL of each washing was taken and added to 9 mL of sterilized physiological saline. The resulting dilution liquid, 1 mL, was taken and added to 9 mL of sterilized physiological saline to prepare a dilution liquid. The prepared dilution liquid was fed in two laboratory dishes, 1 mL each, to which the SCD agar medium was introduced. Culture was carried out at 37°C for 48 hours. The number of colonies was counted after culture to judge the antibacterial effect of the coated test pieces.

The obtained results are shown in Tables 13 to 16. In the tables, "aE + b" represents " $a \times 10^b$."

Table 13
(Vial cell counts in uncoated test pieces immediately after inoculation)

| Sample name | No. of test piece | Lab. dish for cell count | Cell count in lab. dish (number /mL) | Vial cell count in each test piece (number/mL) | Average cell count in uncoated test pieces (number/mL) |
|-----------------|-------------------|--------------------------|--------------------------------------|--|--|
| Uncoated sample | 1 | 1-1 | 401 | 8.5E + 4 | 8.0E + 4 |
| | | 1-2 | 452 | | |
| Uncoated sample | 2 | 2-1 | 417 | 8.3E + 4 | |
| | | 2-2 | 414 | | |
| Uncoated sample | 3 | 3-1 | 355 | 7.2E + 4 | |
| | | 3-2 | 360 | | |

Dilution R was 10-fold when the cell count was measured.

Table 14

(Vial cell counts in uncoated test pieces after 18 hours' culture)

| Sample name | No. of test piece | Lab. dish for cell count | Cell count in lab. dish (number/mL) | Vial cell count in each test piece (number/mL) | Average cell count in uncoated test pieces (number/mL) |
|-----------------|-------------------|--------------------------|-------------------------------------|--|--|
| Uncoated sample | 1 | 1-1 | 261 | 5.8E + 4 | 5.9E + 4 |
| | | 1-2 | 316 | | |
| Uncoated sample | 2 | 2-1 | 265 | 6.0E + 4 | 5.9E + 4 |
| | | 2-2 | 337 | | |

Dilution R was 100-fold when the cell count was measured.

Table 15

(Vial cell counts in coated test pieces after 18 hours' culture)

| Sample name | No. of test piece | Lab. dish for cell count | Cell count in lab. dish (number/mL) | Cell count in test piece (number/mL) | Bacterio-static activity value | Bacterio-cidal activity value | Antiviability |
|--|-------------------|--------------------------|-------------------------------------|--------------------------------------|--------------------------------|-------------------------------|---------------|
| Silver conjugated protein ⑤ Coating weight of 0.15 g/m ² | 1 | 1-1 | 0 | 2E + 3 | 2.5 | 1.6 | 2.5 |
| | | 1-2 | 1 | | | | |
| | 2 | 2-1 | 0 | < 1E + 3 | > 2.8 | > 1.9 | 2.8 |
| | | 2-2 | 0 | | | | |
| | 3 | 3-1 | 16 | 5.5E + 4 | 1.0 | 0.16 | 1.0 |
| | | 3-2 | 39 | | | | |
| | 1 | 1-1 | 0 | 2E + 3 | 2.5 | 1.6 | 2.5 |
| | | 1-2 | 2 | | | | |
| Silver conjugated protein ⑤ Coating weight of 0.45 g/m ² | 2 | 2-1 | 4 | 4E + 3 | 2.2 | 1.3 | 2.2 |
| | | 2-2 | 4 | | | | |
| | 3 | 3-1 | 0 | < 1E + 3 | > 2.8 | > 1.9 | > 2.8 |
| | | 3-2 | 0 | | | | |

(continued Table 15)

| | | | | | | | |
|--|---|-----|----|----------|-------|-------|-------|
| Silver conjugated protein ⑤ Coating weight of 0.90 g/m ² | 1 | 1-1 | 4 | 3E + 3 | 2.3 | 1.4 | 2.3 |
| | | 1-2 | 2 | | | | |
| | 2 | 2-1 | 0 | < 1E + 3 | > 2.8 | > 1.9 | > 2.8 |
| | | 2-2 | 0 | | | | |
| Silver conjugated protein ⑥ 0.15 g/m ² | 3 | 3-1 | 23 | 3.6E + 4 | 1.2 | 0.35 | 1.2 |
| | | 3-2 | 13 | | | | |
| | 1 | 1-1 | 1 | 3E + 3 | 2.3 | 1.4 | 2.3 |
| | | 1-2 | 2 | | | | |
| | 2 | 2-1 | 0 | < 1E + 3 | > 2.8 | > 1.9 | > 2.8 |
| | | 2-2 | 0 | | | | |
| | 3 | 3-1 | 1 | 6E + 3 | 2.0 | 1.1 | 2.0 |
| | | 3-2 | 5 | | | | |

(continued Table 15)

| | | | | | | | |
|---|---|-----|----|----------|-------|-------|-------|
| Silver conjugated protein ⑥ | 1 | 1-1 | 0 | 1E + 3 | 2.8 | 1.9 | 2.8 |
| | | 1-2 | 1 | | | | |
| Coating weight of 0.45 g/m ² | 2 | 2-1 | 0 | 1E + 3 | 2.8 | 1.9 | 2.8 |
| | | 2-2 | 1 | | | | |
| | 3 | 3-1 | 17 | 2.8E + 4 | 1.3 | 0.46 | 1.3 |
| | | 3-2 | 11 | | | | |
| Silver conjugated protein ⑥ | 1 | 1-1 | 0 | < 1E + 3 | > 2.8 | > 1.9 | > 2.8 |
| | | 1-2 | 0 | | | | |
| Coating weight of 0.90 g/m ² | 2 | 2-1 | 0 | < 1E + 3 | > 2.8 | > 1.9 | > 2.8 |
| | | 2-2 | 0 | | | | |
| | 3 | 3-1 | 6 | 8E + 3 | 1.9 | 1.0 | 1.9 |
| | | 3-2 | 2 | | | | |

Dilution R was 100-fold when the cell count was measured.

Table 16

(continued Table 15)

| Sample name | No. of test piece | Lab. dish for cell count | Cell count in lab. dish (number/mL) | Cell count in test piece (number/mL) | Bacterio-static activity value | Bacterio-cidal activity value | Antiviability |
|---------------------------------|-------------------|--------------------------|-------------------------------------|--------------------------------------|--------------------------------|-------------------------------|---------------|
| Silver conjugated protein ⑦ | 1 | 1-1 | 3 | 6E + 3 | 2.0 | 1.1 | 2.0 |
| | | 1-2 | 3 | | | | |
| | 2 | 2-1 | 3 | 7E + 3 | 1.9 | 1.1 | 1.9 |
| Coating | | 2-2 | 4 | | | | |
| weight of 0.15 g/m ² | 3 | 3-1 | 28 | 4.5E + 4 | 1.1 | 0.25 | 1.1 |
| | | 3-2 | 17 | | | | |
| Silver conjugated protein ⑦ | 1 | 1-1 | 52 | 1.5E + 5 | 0.59 | 0.27 | 0.59 |
| | | 1-2 | 99 | | | | |
| | 2 | 2-1 | 3 | 3E + 3 | 2.3 | 1.4 | 2.3 |
| Coating | | 2-2 | 0 | | | | |
| weight of 0.45 g/m ² | 3 | 3-1 | 2 | 5E + 4 | 1.1 | 0.20 | 1.1 |
| | | 3-2 | 3 | | | | |

(continued Table 16)

| | | | | | | | |
|---------------------------------|---|-----|-----|----------|-----|------|-----|
| Silver conjugated protein ⑦ | 1 | 1-1 | 12 | 2.1E + 4 | 1.4 | 0.58 | 1.4 |
| | | 1-2 | 9 | | | | |
| Coating | 2 | 2-1 | 12 | 1.2E + 4 | 1.7 | 0.82 | 1.7 |
| | | 2-2 | 0 | | | | |
| weight of 0.90 g/m ² | 3 | 3-1 | 9 | 2.3E + 4 | 1.4 | 0.54 | 1.4 |
| | | 3-2 | 14 | | | | |
| Not coated | 1 | 1-1 | 261 | 5.8E + 5 | 0 | - | 0 |
| | | 1-2 | 316 | | | | |
| | 2 | 2-1 | 265 | 6.0E + 5 | 0 | - | 0 |
| | | 2-2 | 337 | | | | |

Dilution R was 100-fold when the cell count was measured.

The antibacterial effect was judged by the bacteriostatic activity value, the bacteriocidal activity value and logarithmic reduction rate obtained by the following formulae.

$$\text{Bacteriocidal activity value } L = Ma - Mc$$

5 $\text{Bacteriostatic activity value } S = Mb - Mc$

Ma : Common logarithmic value of the vial cell count (average of three test pieces) on uncoated test pieces immediately after the inoculation

Mb : Common logarithmic value of the vial cell count
10 (average of two test pieces) on uncoated test pieces after 18 hours' culture

Mc : Common logarithmic value of the vial cell count on coated test pieces after 18 hours' culture

$$\text{Logarithmic reduction rate} = \log (B/A) - \log (C/A)$$

15 A : Vial cell count (average of three test pieces) on uncoated test pieces immediately after the inoculation

B : Vial cell count (average of two test pieces) on uncoated test pieces after 18 hours' culture

C : Vial cell count on coated test pieces after 18
20 hours' culture

In most of the test pieces coated with the silver conjugated proteins ⑤ to ⑦, the cell count in the laboratory dishes was suppressed to zero. The bacteriostatic activity value, the bacteriocidal activity value and logarithmic reduction
25 rate were, about two or more, one or more and two or more,

CLAIMS

1. A water-insoluble silver conjugated protein formed of a silver salt and a water-soluble protein containing 0.1 to 200 μ moles of active thiol groups per gram.
- 5 2. A silver conjugated protein as set forth in claim 1, wherein the water-soluble protein is a whey protein or a hydrolysate or a water-solubilized material thereof, or a hydrolysate or a water-solubilized material of an eggshell membrane protein.
- 10 3. A silver conjugated protein as set forth in claim 2, wherein lactose is removed from the whey protein beforehand.
4. A silver conjugated protein as set forth in claim 2, wherein the hydrolysate of the eggshell membrane protein is obtained by an alkali-hydrolysis treatment of eggshell
- 15 membrane.
5. A silver conjugated protein as set forth in claim 1, wherein the silver salt is silver nitrate or silver acetate.
6. A silver conjugated protein as set forth in claim 1, wherein the silver conjugated protein is obtained by a process of
- 20 adding an aqueous solution of the silver salt little by little into an aqueous solution of the protein under stirring, thereby raising the concentration of silver ions in water.
7. A silver conjugated protein as set forth in claim 1, wherein the proportion of the silver salt to the protein in the
- 25 silver conjugated protein is 0.2 to 3 g of the silver salt with

respect to 1 g of the protein.

8. An antibacterial/antifungal agent containing a silver conjugated protein as set forth in any one of claims 1 to 7 as an active component.

5 9. An antibacterial/antifungal paper treated with an
antibacterial/antifungal agent as set forth in claim 8.

10. An antibacterial/antifungal paper as set forth in claim 9, wherein treatment comprises coating.

11. An antibacterial/antifungal paper as set forth in claim
10, wherein a coating weight is 0.01 to 10 g with respect to 1 m²
of paper.

ABSTRACT

According to the present invention, there can be provided a water-insoluble silver conjugated protein formed of a silver salt and a water-soluble protein having an active thiol
5 group content of 0.1 to 200 μ mol/g and also an antibacterial
/antifungal agent and an antibacterial/antifungal paper using
the same, that is, there can be provided a water-insoluble
conjugated protein with a high silver content from which silver
having antibacterial/antifungal activity is not released easily
10 and also an antibacterial/antifungal agent and an antibacterial
/antifungal paper using the same.

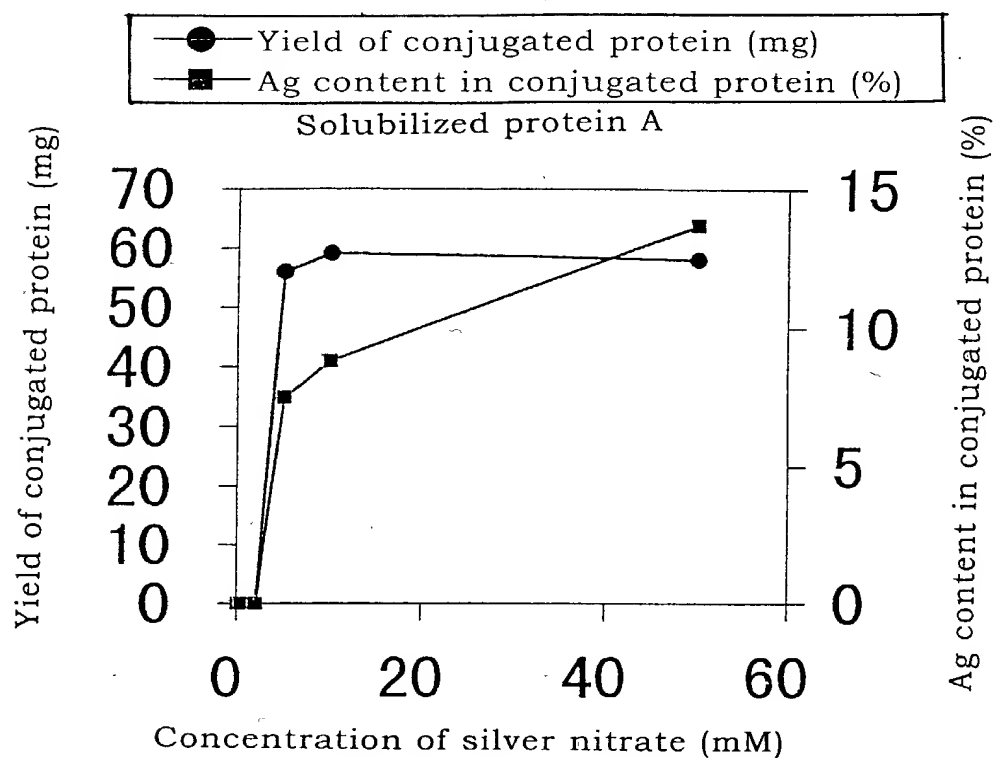


Fig. 1

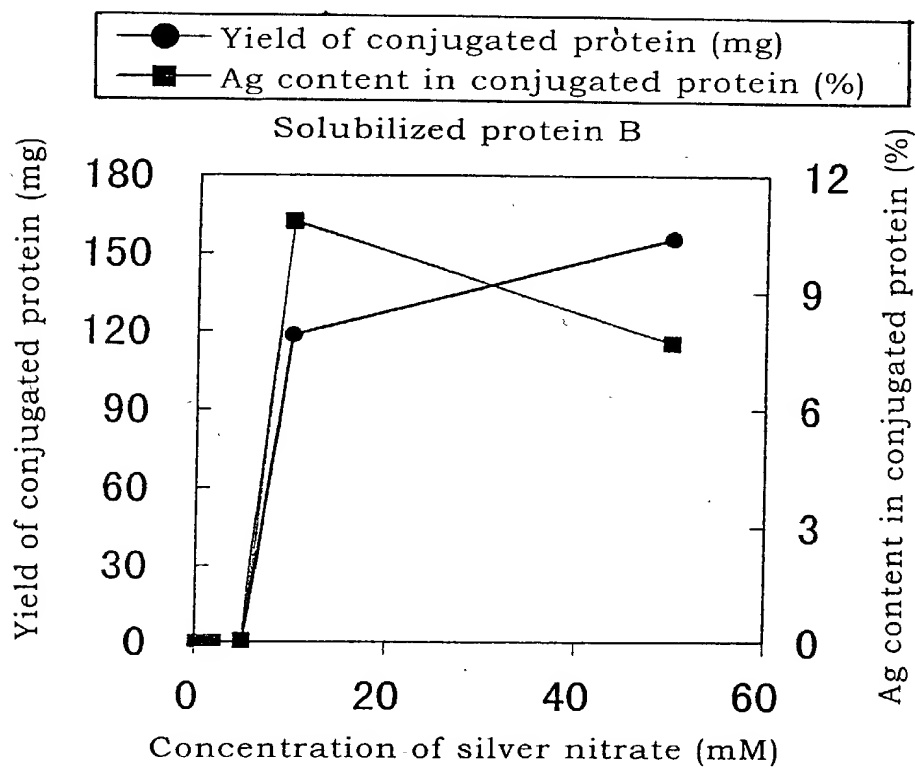
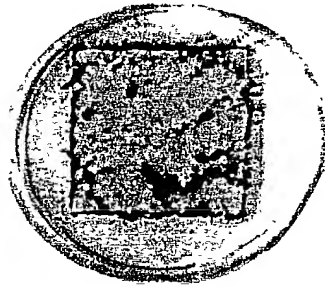


Fig. 2

1008 100 1 0 09/937924

Silver-containing conjugated protein ②



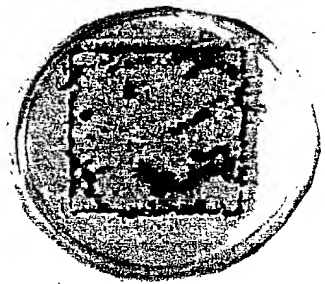
Uncoated

[1]

Coating weight 1g/m²

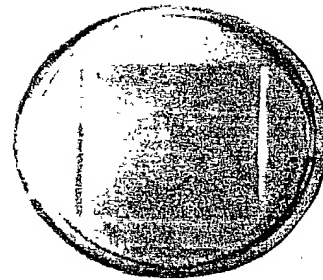
[3]

(a)



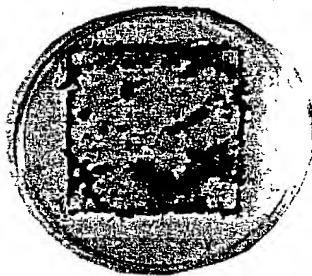
Uncoated

[1]

Coating weight 2g/m²

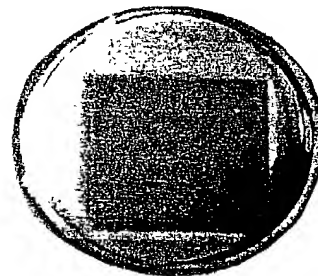
[3]

(b)



Uncoated

[1]

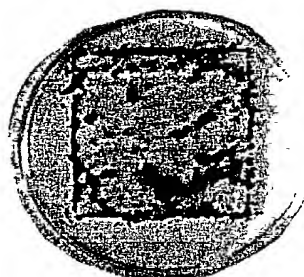
Coating weight 5g/m²

[3]

(c)

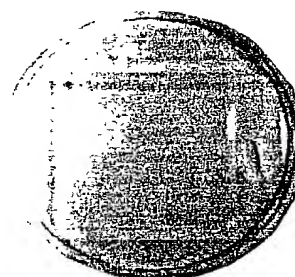
Fig. 3

Silver-containing conjugated protein ③



Uncoated

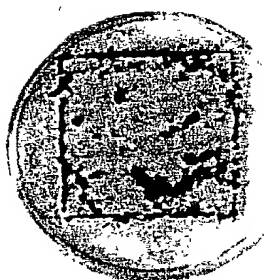
[1]



Coating weight 1g/m²

[3]

(a)



Uncoated

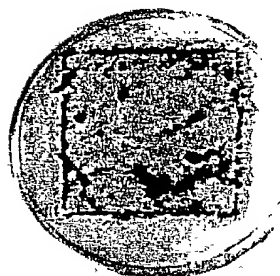
[1]



Coating weight 2g/m²

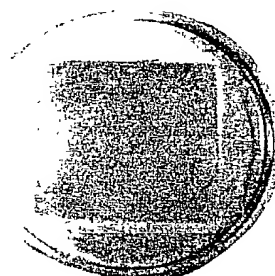
[3]

(b)



Uncoated

[1]



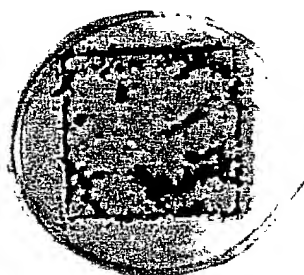
Coating weight 4g/m²

[3]

(c)

Fig. 4

Zeomic



Uncoated

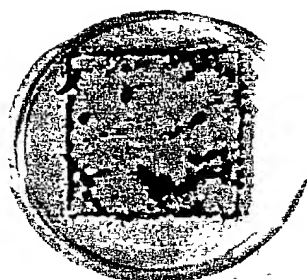
[1]



Coating weight 1g/m²

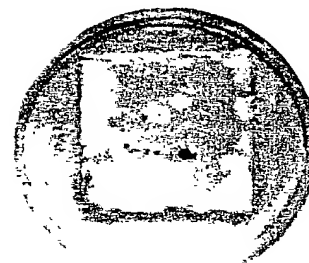
[2]

(a)



Uncoated

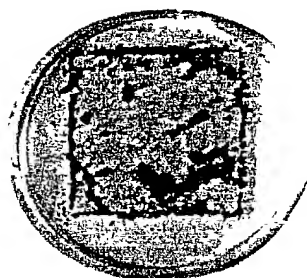
[1]



Coating weight 2g/m²

[2]

(b)



Uncoated

[1]



Coating weight 10g/m²

[3]

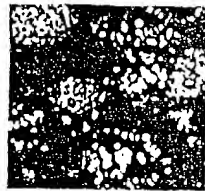
(c)

Fig. 5

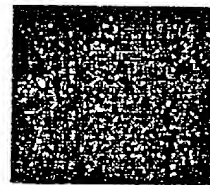
Silver-containing conjugated protein ③

Strain : *Neosartorya fischeri*

Test piece : coated at $1 \mu\text{m}$ per face (0.14 g/m^2)
coating (both faces coated)

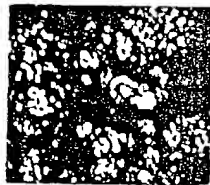


Uncoated



coated

(No. 1)

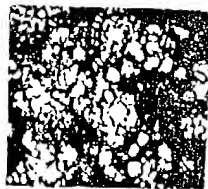


Uncoated

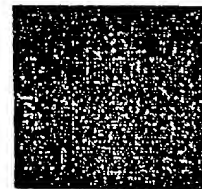


coated

(No. 2)



Uncoated



coated

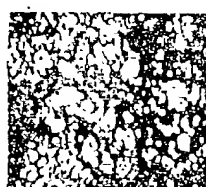
(No. 3)

Fig. 6

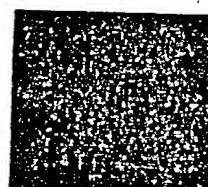
Silver-containing conjugated protein ③

Strain : *Neosartorya fischeri*

Test piece : coated at $3 \mu\text{m}$ per face (0.42 g/m^2)
coating (both faces coated)

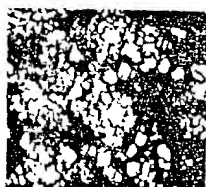


Uncoated^s

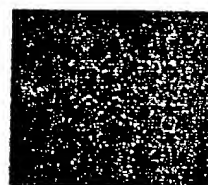


coated

(No. 1)

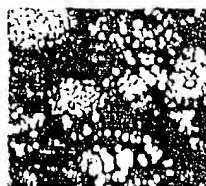


Uncoated



coated

(No. 2)



Uncoated



coated

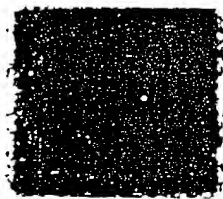
(No. 3)

Fig. 7

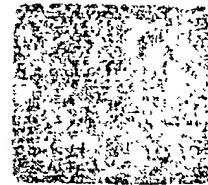
Silver-containing conjugated protein ③

Strain : 5 strains according to JIS

Test piece : coated at $1 \mu\text{m}$ per face (0.14 g/m^2)
coating (both faces coated)



Uncoated

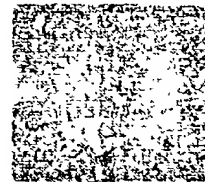


coated

(No. 1)



Uncoated

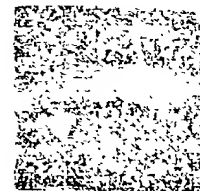


coated

(No. 2)



Uncoated



coated

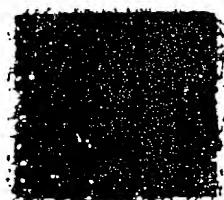
(No. 3)

Fig. 8

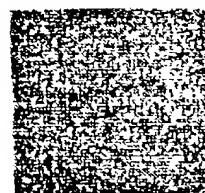
Silver-containing conjugated protein ③

Strain : 5 strains according to JIS

Test piece : coated at $3 \mu\text{m}$ per face (0.42 g/m^2)
coating (both faces coated)



Uncoated

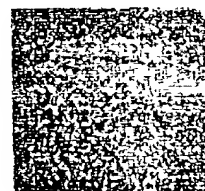


coated

(No. 1)



Uncoated

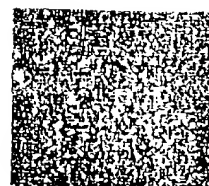


coated

(No. 2)



Uncoated

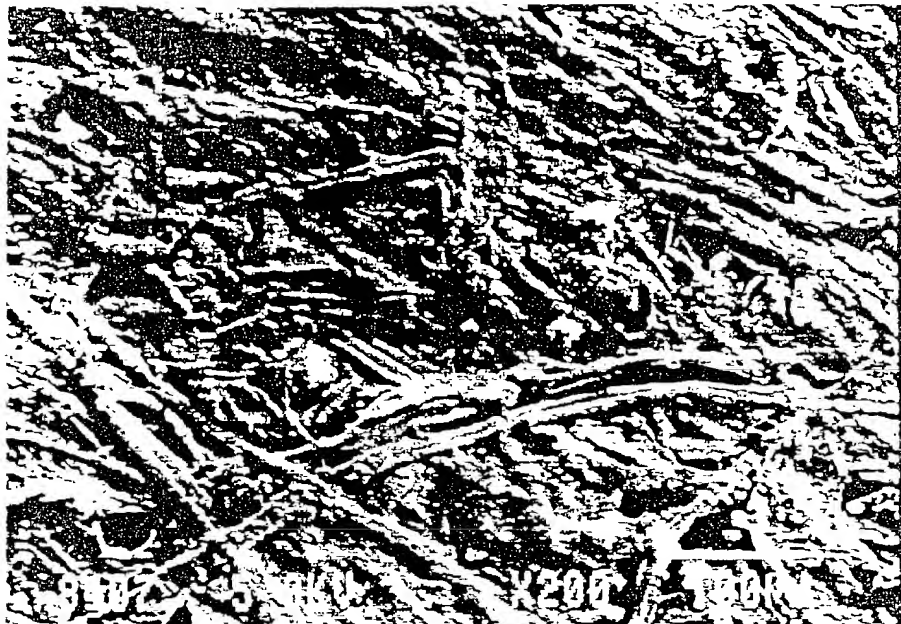


coated

(No. 3)

Fig. 9

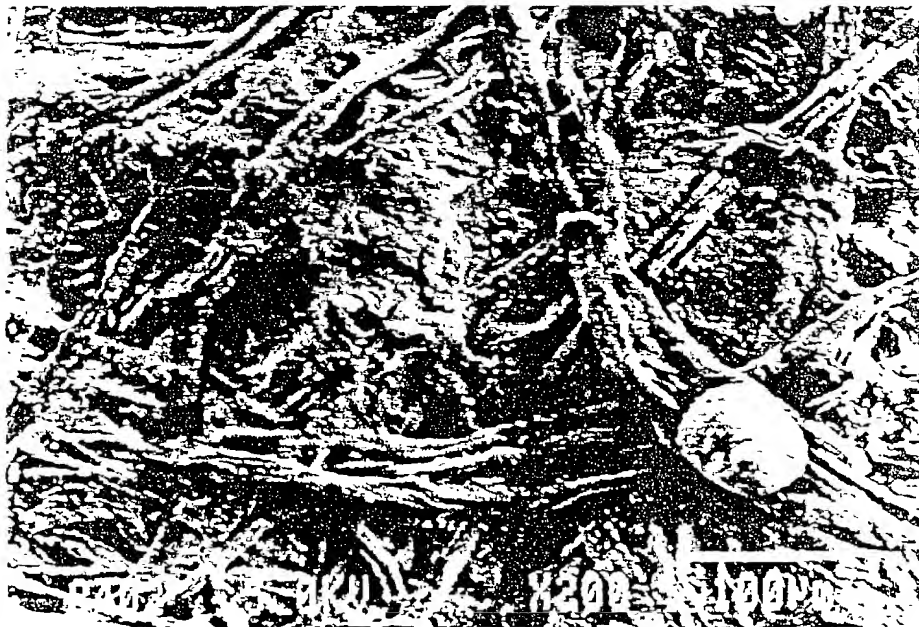
Silver-containing conjugated protein ① coated copy paper



100 μ m

Fig. 10

Silver-containing conjugated protein ③ coated copy paper




100 μ m

Fig. 11

| 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | | 10 | | 11 | | 12 | | 13 | | 14 | | 15 | | 16 | | 17 | | 18 | | 19 | | 20 | | 21 | | 22 | | 23 | | 24 | | 25 | | 26 | | 27 | | 28 | | 29 | | 30 | | 31 | | 32 | | 33 | | 34 | | 35 | | 36 | | 37 | | 38 | | 39 | | 40 | | 41 | | 42 | | 43 | | 44 | | 45 | | 46 | | 47 | | 48 | | 49 | | 50 | | 51 | | 52 | | 53 | | 54 | | 55 | | 56 | | 57 | | 58 | | 59 | | 60 | | 61 | | 62 | | 63 | | 64 | | 65 | | 66 | | 67 | | 68 | | 69 | | 70 | | 71 | | 72 | | 73 | | 74 | | 75 | | 76 | | 77 | | 78 | | 79 | | 80 | | 81 | | 82 | | 83 | | 84 | | 85 | | 86 | | 87 | | 88 | | 89 | | 90 | | 91 | | 92 | | 93 | | 94 | | 95 | | 96 | | 97 | | 98 | | 99 | | 100 | |
|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|----|--|-----|--|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

09/937924

Zeom i c coated copy paper



1 0 0 μm

Fig. 12

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| | First Named Inventor | Yoshihiro Kawaguchi |
| | COMPLETE IF KNOWN | |
| | Application Number | 09 / 937,924 |
| | Filing Date | October 2, 2001 |
| | Group Art Unit | |
| Examiner Name | | |

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SILVER CONJUGATED PROTETIN, AND ANTIBACTERIAL/ANTIFUNGAL AGENT AND ANTIBACTERIAL/ANTIFUNGAL PAPER USING THE SAME

the specification of which *(Title of the Invention)*

☐ is attached hereto OR ☒ was filed on (MM/DD/YYYY) **March 30, 2000** as United States Application Number or PCT International Application Number **PCT/JP00/02053** and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

| Prior Foreign Application Number(s) | Country | Foreign Filing Date (MM/DD/YYYY) | Priority Not Claimed | Certified Copy Attached? | |
|-------------------------------------|---------|----------------------------------|--------------------------|--------------------------|-------------------------------------|
| | | | | YES | NO |
| HEI 11 (1999)-096847 | Japan | April 2, 1999 | <input type="checkbox"/> | <input type="checkbox"/> | <input checked="" type="checkbox"/> |
| 2000-030690 | Japan | February 8, 2000 | <input type="checkbox"/> | <input type="checkbox"/> | <input checked="" type="checkbox"/> |

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

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| Application Number(s) | Filing Date (MM/DD/YYYY) |
|-----------------------|--------------------------|
| | |

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(Page 1 of 3E)

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| U.S. Parent Application or PCT Parent Number | Parent Filing Date (MM/DD/YYYY) | Parent Patent Number (if applicable) |
|--|---------------------------------|--------------------------------------|
| PCT/JP00/02053 | 03/30/2000 | |

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

| | |
|--|------------------------|
| Given Name (first and middle (if any)) | Family Name or Surname |
| YOSHIHIRO | KAWAGUCHI |

| | | | | | |
|----------------------|---|-------|-------|---------|---------------|
| Inventor's Signature | <i>Yoshihiro Kawaguchi</i> | | | Date | March 1, 2002 |
| Residence: City | Osaka-shi, Osaka | State | Japan | Country | JP |
| Post Office Address | c/o Katayama Chemical, Inc., 2-10-15, Higashiawaji, | | | | |
| Post Office Address | Higashiyodogawa-ku, Osaka-shi, Osaka 533-0023 | | | | |
| City | Osaka | State | Japan | ZIP | 533-0023 |
| | | | | Country | JP |

☒ Additional inventors are being named on the _____ supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

DOCKET NO. 10690/126885

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